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INFECTIONS IN MICE WITH TACHYZOITES AND BRADYZOITES OF *NEOSPORA CANINUM* (PROTOZOA: APICOMPLEXA)

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ABSTRACT: Tachyzoites of 2 isolates of *Neospora caninum* (NC-1 and NC-2) were inoculated subcutaneously (s.c.), intraperitoneally (i.p.), or orally into mice to compare the effects of route of inoculation on pathogenicity. Mice developed more severe disease, and disease occurred sooner when inoculated with the NC-1 isolate compared to the NC-2 isolate. Deaths occurred earlier in mice inoculated i.p. with either isolate. Mice inoculated orally or s.c. with tachyzoites responded similarly to infection. Tissue cysts of the NC-2 isolate produced infections in mice following oral or s.c. inoculation. Lesions seen in mice inoculated with tachyzoites or bradyzoites were primarily acute pneumonia, myositis, encephalitis, ganglioradiculoneuritis, and pancreatitis. In vitro studies demonstrated that tachyzoites of both isolates were killed by incubation in pepsin-HCl solution but not 1% trypsin solution. Bradyzoites of the NC-2 isolate were able to withstand treatment with pepsin-HCl solution.

Neospora caninum is an apicomplexan parasite that causes severe disease in dogs (Cummings et al., 1988; Dubey and Beattie, 1988; Dubey, Carpenter et al., 1988; Dubey, Hattel et al., 1988; Dubey and Lindsay, 1989a; Hay et al., 1990). It was first described and isolated from naturally infected dogs in 1988 (Dubey, Carpenter et al., 1988; Dubey, Hattel et al., 1988). Until that time it had been confused with the structurally similar parasite *Toxoplasma gondii* (Dubey and Beattie, 1988; Dubey, Carpenter et al., 1988). Canine neosporosis is characterized by encephalitis, polyradiculoneuritis, polymyositis, and ascending paralysis.

Many aspects of the life cycle and sources of infection are not known. Tachyzoites, rapidly dividing stages, are found in many types of cells in many tissues. Tissue cysts contain bradyzoites, slowly multiplying stages, and are found only in the central nervous system (CNS). The parasite can be transmitted transplacentally (Dubey, Hattel et al., 1988; Dubey and Lindsay, 1989a, 1989b). Tachyzoites and tissue cysts develop in extraintestinal tissues of animals. These similarities to *T. gondii* indicate that other stages of a coccidian life cycle might develop in the intestines of a yet unknown definitive host.

A cat fed tissues containing *N. caninum* cysts and tachyzoites became infected (Dubey and Lindsay, 1989b). Whether the infection occurred via the buccal mucosa or via stages surviving passage through the stomach is not known, nor is the stage responsible for initiating the infection. However, this does demonstrate that stages of *N. caninum* are infective following ingestion.

Experimental infections in methylprednisolone acetate (MPA)-treated mice have provided a laboratory model to study aspects of the transmission, pathogenesis, and chemotherapy of *N. caninum* infections (Lindsay and Dubey, 1989b). The mouse model was developed using subcutaneous (s.c.) inoculation of tachyzoites. Detailed experiments on the infectivity of *N. caninum* tissue cysts and effects of route of inoculation of tachyzoites on infectivity and pathogenesis have not been reported.

In the present study, we examined the effects of oral, s.c., and intraperitoneal (i.p.) inoculation of tachyzoites of 2 different isolates of *N. caninum*, and oral and s.c. inoculation of bradyzoites of 1 isolate in MPA-treated mice. We also conducted in vitro studies to determine the resistance of tachyzoites and bradyzoites to the digestive enzymes, pepsin and trypsin.

MATERIALS AND METHODS

Parasites and preparation of inocula

Tachyzoites of the NC-1 (Dubey, Hattel et al., 1988) and NC-2 (Hay et al., 1990) isolates of *N. caninum* were isolated originally from naturally infected puppies. Both isolates are maintained by continuous passage in bovine monocyte (BM) cell cultures (Lindsay and Dubey, 1989a) that are grown in culture medium consisting of RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml dihydrostreptomycin, 50 U/ml penicillin G (GIBCO, Grand Island, New York), and 5×10^{-3} 2-mercaptoethanol (Sigma Chemical Company, St. Louis, Missouri). To obtain tachyzoites from host cells, monolayers were scraped off the plastic growth surface, which ruptures most in-

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EXHIBIT B

fected BM cells, and the culture medium was then filtered through a sterile 3-μm filter. Tachyzoites were counted in a hemacytometer, and the volume of solution was adjusted with Hanks' balanced salt solution (HBSS) so that 1 ml contained 2×10^5 tachyzoites for s.c. inoculations and 0.5 ml contained 2×10^5 tachyzoites for oral or i.p. inoculations.

Bradyzoites were obtained from tissue cysts in the brains of 2 mice inoculated 6 mo previously with the NC-2 isolate. One mouse had mild posterior paralysis, and tissue cysts were identified in a brain smear. The other mouse had no clinical sign, and tissue cysts were not seen in a brain smear. The brains of both mice were triturated together in HBSS using a mortar and pestle, and a portion of the mixture was digested in pepsin-HCl solution (pH 0.8) (Dubey and Beattie, 1988) for 10 min. The digested mixture was washed 3 times in cell culture medium by centrifugation prior to use. Portions of undigested brain mixture were used for oral inoculations, and portions of the digested mixture were used for oral and s.c. inoculations. The number of cysts or bradyzoites that each mouse received was not determined.

Inoculation and examination of mice

Female Swiss white mice (20–25 g) were used for inoculations. The number of mice inoculated, the isolate used for inoculation, and the route of inoculation are given in Table I. All mice inoculated with *N. caninum* were given intramuscular injections of 4 mg MPA (Med-Tech Inc., Elwood, Kansas) on days −7, 0, and 7 postinoculation (PI) of parasites to facilitate detection of *N. caninum* stages (Lindsay and Dubey, 1989b). Control groups consisted of mice that were given only MPA and mice that were not given MPA or tachyzoites.

Tissues were collected for histological examination from mice that were killed while moribund, mice judged to be recently dead, and all mice that were killed when the study was terminated 28 days PI. Portions of brain, spinal column, tongue, lung, heart, diaphragm, thigh muscle, pancreas, adrenal, kidneys, and liver were fixed in neutral buffered 10% formalin and processed for light microscopic examination after staining with hematoxylin and eosin. Fresh smears were made from the lungs of mice that died and were examined immediately for tachyzoites with light microscopy.

In vitro assay for effects of pepsin-HCl and trypsin solutions on parasites

Tachyzoites of *T. gondii* survive in trypsin but not in pepsin solutions, whereas bradyzoites survive in both solutions (Jacobs et al., 1960). To determine the effects of these digestive enzymes on *N. caninum*, monolayers of infected BM cells containing tachyzoites of either the NC-1 isolate or the NC-2 isolate were scraped from the plastic growth surface, pelleted by centrifugation, and exposed to pepsin-HCl solution (pH 0.8) or 1% (w/v) trypsin in HBSS (pH 5.5) for 30 min. The samples were then washed by centrifugation in cell culture medium and inoculated onto monolayers of uninfected BM cells. Portions of the undigested and pepsin-HCl-digested brain mixture were processed and inoculated similarly. Cell cultures were then examined for 30 days for growth of *N. caninum* (Lindsay and Dubey, 1989a).

TABLE I. Protocol for inoculation of mice with tachyzoites (T) or bradyzoites (B) of *Neospora caninum* and results of inoculations.

Group	Isolate	Mode*	Digested	MPA†	No. infected/ no. inoculated
1	NA‡	NA	NA	None	0/5
2	NA	NA	NA	Yes	0/10
3	NC-1	s.c.-T	No	Yes	10/10
4	NC-1	i.p.-T	No	Yes	10/10
5	NC-1	O-T	No	Yes	10/10
6	NC-1	O-T	No	Yes	10/10
7	NC-2	s.c.-T	No	Yes	5/5
8	NC-2	i.p.-T	No	Yes	10/10
9	NC-2	O-T	No	Yes	4/5
10	NC-2	s.c.-B	Yes	Yes	10/10
11	NC-2	O-B	Yes	Yes	7/10
12	NC-2	O-B	No	Yes	1/5

* s.c., subcutaneous inoculation; i.p., intraperitoneal inoculation; O, oral inoculation.
† Methylprednisolone acetate.
‡ Not applicable.

RESULTS

In vivo experiments

Mice inoculated s.c. with tachyzoites of either isolate developed acute neosporosis, and all were infected when examined at necropsy (Table I). However, differences in the responses of mice to s.c. inoculation of the 2 strains were noted. All 10 mice inoculated s.c. with the NC-1 isolate (group 3) developed pneumonia and died or were killed when moribund 7–11 days PI, whereas 3 of 5 mice inoculated s.c. with the NC-2 isolate (group 7) died 15–17 days PI. One of the remaining mice in group 7 had a paralyzed right hind leg, and the other mouse was normal when killed 28 days PI. Lesions seen in the tissues of mice in group 3 consisted mainly of acute pneumonia. Encephalitis, pneumonia, myositis, myocarditis, and pancreatitis were seen in the tissues of the 3 mice in group 7 that died 15–17 days PI. The 2 surviving mice in group 7 had lesions in the brain and spinal cord that consisted of vasculitis, gliosis, and mineralization. Ganglioradiculoneuritis also was seen in sections of the spinal columns of these mice.

Mice inoculated i.p. with tachyzoites of either isolate all became infected (Table I). Five of 10 mice inoculated i.p. with the NC-1 isolate (group 4) died 4 days PI and the remaining 5 were moribund and killed on this day. Mice inoculated i.p. with the NC-2 isolate (group 8) reacted similarly to infection; 1 died 6 days PI and 2 were killed when moribund on this day. Two group 8 mice died 7 days PI and the remaining 5 were

killed on this day. Minimal amounts (0.3–0.8 ml) of yellowish ascites fluid were present in the abdominal cavities of mice in groups 4 and 8. Microscopic examination of the ascites fluid revealed many inflammatory cells but few tachyzoites. Lesions were confined to the visceral tissues. The most severe microscopic lesions were seen in the pancreas of infected mice and consisted of diffuse necrosis of acinar cells (mainly at the margins of pancreatic lobes) and infiltration of pancreatic connective tissue by inflammatory cells. Minimal lesions were seen also in the livers, adrenal glands, and abdominal diaphragm of i.p.-inoculated mice and consisted of small areas of necrosis or myositis.

Mice inoculated orally with tachyzoites of either *N. caninum* isolate became infected (Table I) and isolate-related differences in responses were observed. Fourteen of 20 mice inoculated orally with the NC-1 isolate (groups 5 and 6) died of acute pneumonia 7–12 days PI, 2 died from encephalitis 26 days PI, 1 died from encephalitis 28 days PI, and 3 were killed 28 days PI at the end of the study. One of the 3 surviving mice had a head tilt to the right indicating encephalitis; the other 2 appeared normal. None of 5 mice inoculated orally with the NC-2 isolate developed clinical neosporosis or died. However, 4 of these mice had lesions in the brain consistent with neosporosis, and tachyzoites were seen in 2 of these 4 mice. Microscopic lesions seen in orally inoculated mice were similar to s.c.-inoculated mice examined at the same day PI.

Subcutaneous inoculation of pepsin-digested brain mixture of the NC-2 isolate (group 10) produced infections in 10 of 10 mice (Table I). Four mice died 14–16 days PI, and the remaining 6 mice were killed 28 days PI. Three of the 6 mice had clinical signs; 2 had head tilts and 1 had hind leg paralysis. Microscopic lesions were present in the 6 mice and were similar to those seen in mice inoculated with tachyzoites.

Oral inoculation of mice with brain mixture of the NC-2 isolate produced infections in the mice (Table I). Oral inoculation of undigested brain material (group 12) produced infection in only 1 of 5 mice. The infected mouse died 14 days PI and had mild myositis and severe pneumonia. However, fungal hyphae were also seen in the lungs of this mouse, and its death can not be attributed to *N. caninum*-induced pneumonia. Oral inoculation of mice with pepsin-digested brain containing cysts produced infections in 7 of 10 mice (group 11). One of the 10 mice died 17 days PI; the other 9 were killed 28 days PI. Pneumonia and encephalitis were present in the mouse that died. None of the 9 mice had clinical signs when killed. The 6 infected mice killed 28 days PI had lesions of encephalitis and ganglioradiculoneuritis. Tachyzoites were seen in the brains of 2 of these 6 mice. Lesions were similar to those of other *N. caninum*-infected mice.

Neospora caninum tissue cysts were not observed in any mouse in the present study. Mice that did not receive tachyzoites or MPA (group 1) and mice that received only MPA (group 2) remained healthy during the study. Mice in group 2 usually had small or inapparent thymuses and mesenteric lymph nodes. No microscopic lesion was seen in the tissues of mice in groups 1 or 2.

In vitro experiments

Bovine monocyte cell cultures that were inoculated with undigested and digested brain material containing cysts of the NC-2 isolate became infected. This indicates that cysts were present in the undigested inoculum and that bradyzoites can survive digestion in pepsin-HCl solution. Exposure of tachyzoites of both isolates to pepsin-HCl solution was lethal; none of the BM cell cultures became infected. Exposure of tachyzoites of both isolates to 1% trypsin solution was nonlethal; both isolates were infectious for BM cells after incubation.

DISCUSSION

The pathogenicity of the NC-1 isolate has been characterized previously in MPA-treated mice following s.c. inoculation (Lindsay and Dubey, 1989b). The present study provides information on oral and i.p.-induced infections. It demonstrated that mice are susceptible to oral inoculation with tachyzoites and that the infections produced were similar to those produced by s.c. inoculation. Intraperitoneal inoculation of tachyzoites was found to differ from both s.c. and oral routes of inoculation in that mice died earlier in the infection, before pneumonia, myositis, or encephalitis developed.

The NC-1 isolate appears to be more pathogenic for mice than does the NC-2 isolate. Disease developed later, was less severe, and more mice survived to the end of the study when inoculated with tachyzoites of the NC-2 isolate.

Bradyzoites from tissue cysts of the NC-2 isolate were shown to be infective following oral or s.c. inoculation. The resulting infections were

similar to tachyzoite-induced infections. Not all mice inoculated orally with tissue cysts became infected. The low infectivity of undigested brain mixture is probably related to the low numbers and uneven distribution of tissue cysts in the inoculum.

Results of the in vitro studies to characterize the resistance of tachyzoites to pepsin-HCl and trypsin solutions indicated that pepsin-HCl was lethal, whereas trypsin solution was not. This is also true for tachyzoites of *T. gondii* (Jacobs et al., 1960). The inability of *N. caninum* tachyzoites to withstand pepsin-HCl solution also indicates that cysts are not present in the cell cultures because *N. caninum* bradyzoites were not killed by incubation in pepsin-HCl solution. The tissue cyst wall of *T. gondii* is destroyed soon after incubation in pepsin solution, and the released bradyzoites can survive for several hours (Jacobs et al., 1960). We did not examine the effect of pepsin solution on the tissue cyst wall of *N. caninum*.

The most puzzling aspect of the present study is the infectivity of orally inoculated tachyzoites. Results of in vitro studies showed that tachyzoites were killed by pepsin-HCl solution, indicating that they should not have survived passage through the stomach. There are 2 possible explanations for the observed results: (1) tachyzoites are infective by oral inoculation and can survive passage through the stomach, and (2) during inoculation the tachyzoites can penetrate the mucosa of the oral cavity/esophagus, bypass the stomach, and produce infection. The latter route of infection might be aided by any trauma to the mucosa resulting from inoculation.

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